

Novel Polyphenol Oxidase Mined from a Metagenome Expression Library of Bovine Rumen

BIOCHEMICAL PROPERTIES, STRUCTURAL ANALYSIS, AND PHYLOGENETIC RELATIONSHIPS^{*[5]}

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RL5, a gene coding for a novel polyphenol oxidase, was identified through activity screening of a metagenome expression library from bovine rumen microflora. Characterization of the recombinant protein produced in *Escherichia coli* revealed a multipotent capacity to oxidize a wide range of substrates (syringaldazine > 2,6-dimethoxyphenol > veratryl alcohol > guaiacol > tetramethylbenzidine > 4-methoxybenzyl alcohol > 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) >> phenol red) over an unusually broad range of pH from 3.5 to 9.0. Apparent K_m and k_{cat} values for ABTS, syringaldazine, and 2,6-dimethoxyphenol obtained from steady-state kinetic measurements performed at 40 °C, pH 4.5, yielded values of 26, 0.43, and 0.45 μM and 18, 660, and 1175 s^{-1} , respectively. The K_m values for syringaldazine and 2,6-dimethoxyphenol are up to 5 times lower, and the k_{cat} values up to 40 times higher, than values previously reported for this class of enzyme. RL5 is a 4-copper oxidase with oxidation potential values of 745, 400, and 500 mV versus normal hydrogen electrode for the T1, T2, and T3 copper sites. A three-dimensional model of RL5 and site-directed mutants were generated to identify the copper ligands. Bioinformatic analysis of the gene sequence and the sequences and contexts of neighboring genes suggested a tentative phylogenetic assignment to the genus *Bacteroides*.

Kinetic, electrochemical, and EPR analyses provide unequivocal evidence that the hypothetical proteins from *Bacteroides thetaiotaomicron* and from *E. coli*, which are closely related to the deduced protein encoded by the RL5 gene, are also multicopper proteins with polyphenol oxidase activity. The present study shows that these three newly characterized enzymes form a new family of functional multicopper oxidases with laccase activity related to conserved hypothetical proteins harboring the domain of unknown function DUF152 and suggests that some other of these proteins may also be laccases.

Laccases are multicopper oxidoreductases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) able to oxidize a wide variety of phenolic and nonphenolic compounds, including industrial dyes, polycyclic aromatic hydrocarbons, pesticides, and alkenes, but also capable of performing polymerization, depolymerization, methylation, and demethylation reactions (Refs. 1–8; see extensive reviews in Refs. 2 and 5). They are useful enzymes for a variety of applications, including decolorization of different types of recalcitrant dyes (9), bioremediation of soils and water (10, 11), and kraft pulp biobleaching (12), as well as in other biotechnological applications (for review see Ref. 10), and there is considerable interest to find novel proteins with laccase-like activity through the exploration of biological diversity. Laccases are widely distributed among prokaryotes and eukaryotes (Refs. 13–18; for review see Refs. 14–17), and structural and comparative studies (19–24) have identified conserved regions in which histidine residues can bind four copper atoms located at two main sites that are involved in catalytic activity (for review see Ref. 5). The T1 copper site, which has the highest potential, is assumed to be the first electron acceptor. The other three copper ions form a cluster in two adjacent sites, designated T2/T3, one in the T2 site and two in the binuclear T3 site. Electrons captured by the T1 site are transferred via T2/T3 to the product, leading to

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S5 and annex DNA and amino acid sequences.

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product oxidation and reduction of oxygen to water (for review see Refs. 5 and 25).

Microbial diversity constitutes a largely unexplored treasure chest of new enzymes that can be exploited in biocatalytic processes. At present, because of the inability to cultivate most microbes, the only means of accessing the resources of the microbial world is to harvest genetic resources in so-called metagenome libraries (for review see Refs. 26 and 27). We recently showed that a metagenome library of bovine rumen microflora is a rich source of new microbial diversity and, more interestingly, that a significant proportion of hydrolases retrieved from the library has sequences with very low homology to known proteins having the same function (28) or even improved activities in comparison with known counterparts (29). The rumen ecosystem represents an anaerobic or microaerophilic environment characterized by high degradation and turnover rates of plant polymer substrates. Along with a number of fibrolytic enzymes needed to degrade components of plant cell walls (such as hemicellulases, xylases, β -xylanases, arabinofuranosidases, cellulases, glucanohydrolases, glucosidases, and endoglucanases), laccases (phenol oxidases) and peroxidases (lignin peroxidases) are important plant polymer-modifying enzymes that facilitate digestion of lignin (30). In this article, we report on the retrieval from a metagenome expression library of bovine rumen and the characterization of a new polyphenol oxidase with laccase activity, designated hereinafter as RL5. This laccase is unusual in three respects: it lacks any sequence relatedness to the known laccases but shows homology to a large protein family, DUF152; it exhibits much higher activity and substrate affinities than thus far described in laccases; and it represents the first functionally characterized member of a new laccase family. In this study, we also cloned and characterized two other homologous representatives of DUF152, namely BT4389 and YifH proteins from *Bacteroides thetaiotaomicron* and *Escherichia coli*, correspondingly, and found that both exhibit structural, catalytic, and spectral properties typical of multicopper oxidases. The present study suggests that other highly homologous conserved hypothetical proteins harboring the domain of unknown function, DUF152, may thus be functional polyphenol oxidases.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—ABTS,³ DMP, 4-hydroxybenzyl alcohol, veratryl alcohol, guaiacol, 1-HBT, tetramethylbenzidine, poly-R 478, and violuric acid were purchased from Sigma, and 3,4-dimethoxybenzyl alcohol, SGZ, and phenol red were from Aldrich. All chemicals were of analytical grade. Molecular mass markers for SDS-PAGE were obtained from Novagen (Madison, WI), restriction and modifying enzymes were from New England Biolabs (Beverly, MA), and DNase I grade II was from Roche Applied Science. Chromatographic media were from Amersham Biosciences. DNA manipulations were carried out according to the protocols of Sambrook *et al.* (31) and according to the manufacturers' instructions for the enzymes and materials employed.

³ The abbreviations used are: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DMP, 2,6-dimethoxyphenol; DUF, domain of unknown function; ICP-MS, high-resolution inductively coupled plasma mass spectrometry; 1-HBT, 1-hydroxybenzotriazole; NHE, normal hydrogen electrode; SGZ, syringaldazine; ORF, open reading frame.

Bacterial Strains—*E. coli* strains XL1-Blue MRF' (for library construction and screening) and XLOLR (for expression of the laccase from phagemid) (both from Stratagene, La Jolla, CA), TOP 10 (for site-directed mutagenesis and expression of mutants; Invitrogen), and BL21(DE3) Origami (pLysS) (Novagen) were maintained and cultivated according to the recommendations of suppliers and standard protocols described elsewhere (31).

Retrieval of the RL5 Laccase Gene—The bacteriophage λ -based expression library of DNA extracted from cow rumen fluid has been described elsewhere (28). Laccase production by library clones was screened by plating hybrid phage-infected cells on seeded NZY soft agar containing 50 μ M syringaldazine. A pBK-cytomegalovirus phagemid, designated pBKRL5, was produced from one laccase-positive phage (identified by the presence of a purple halo, produced by the oxidation of SGZ, on agar plate) by co-infection with a helper phage according to the Stratagene protocol. The DNA insert was sequenced from both ends using universal primers and primer walking.

Site-directed Mutagenesis—The RL5 laccase mutant variants were prepared using a QuikChange XL[®] site-directed mutagenesis kit (Stratagene), according to the vendor's instructions, with appropriate oligonucleotides and the phagemid pBKRL5 as template. Plasmids containing mutant RL5 gene variants were introduced into *E. coli* TOP10 (Invitrogen) by electroporation. Plasmids containing wild type and mutant genes were sequenced at the Sequencing Core Facility of the Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas (Madrid), using the Applied Biosystems 377 automated fluorescent DNA sequencer. The sequencing primer used was as follows: F1, 5'-ATA GAA CTT GAG AAA TTG GAT TTT GC-3'.

Purification of RL5 Variants—Genes of the full-length wild type and mutant RL5 proteins were amplified and fused with a hexahistidine His₆ tag at the C terminus as follows. Genes were PCR-amplified from pBKRL5 plasmids using the oligonucleotide primer sequences for RL5F-Nde (5'-GAA GGA GAT ATA CAT ATG ATA GAA CTT GAG AAA TTG GAT TTT GC-3') and RL5R-Kpn (5'-TGG TAC CTT AGT GGT GGT GGT GGT GGT GTT TCC TAT ATA TTC CGG TGA AGG TGC G-3') (underlined are the nucleotides introduced for the histidine tag). Reactions were carried out in a total volume of 50 μ l in the presence of 2 units of *Taq* polymerase (Qiagen, Hilden, Germany) for 1 min at 94 °C followed by 25 cycles of 20 s at 94 °C, 60 s at 40 °C, and 1 min at 72 °C and a final elongation step for 5 min at 72 °C and 15 min at 10 °C. The amplicons were purified by electrophoresis on a 0.75% agarose gel and cloned into the pCR2.1 plasmid (Invitrogen), and hybrid plasmids electroporated into *E. coli* TOP10 cells (Invitrogen), as recommended by the supplier, were then plated on Luria-Bertani (LB) agar supplemented with kanamycin (50 μ g/ml).

Transformant clones were cultured in LB supplemented with 50 μ g/ml kanamycin and different concentrations of CuSO₄ ranging from 0.1 to 1 mM at 37 °C in a 1-liter shaken flask. It was found that 500 μ M CuSO₄ gave enzyme with the highest activity, with 10 μ M giving only 10% and 1 mM giving less than 40%. When the cultures reached an A₆₀₀ of 0.6, 1 mM isopropyl- β -D-galactopyranoside was added, and incubation continued for 4 h.

Cells were then collected by centrifugation (30 min, $8,000 \times g$, 4°C) and resuspended in 50 mM NaH_2PO_4 , pH 6.1, 150 mM NaCl, and 20 mM imidazole, after which lysozyme was added (1 mg/ml). Cell suspensions were incubated on ice for 30 min and then sonicated four times for 30 s. Cell lysates were centrifuged for 20 min at 4°C , $25,000 \times g$, and the His₆-tagged enzymes were purified at 25°C on a 1-ml HisTrap HP column (Amersham Biosciences). After the columns were washed with 4 ml of 20 mM NaH_2PO_4 , pH 7.4, 150 mM NaCl, and 20 mM imidazole, recombinant enzymes were eluted with 10 ml of 20 mM NaH_2PO_4 , pH 7.4, 150 mM NaCl, and 500 mM imidazole. The monitoring of enzyme activity during purification was carried out by spectrophotometric measurement of oxidation of SGZ at 530 nm. SDS-PAGE was performed on 12% (v/v) acrylamide gels, as described by Laemmli (32), in a Bio-Rad Mini Protean system. Protein concentrations were determined according to Bradford (33) with bovine serum albumin as standard.

Cloning and Purification of Hypothetical Proteins from *B. thetaiotaomicron* and *E. coli*—Genes of the full-length hypothetical protein from *B. thetaiotaomicron* DSM 2079 (BT4389) and *E. coli* K-12 (YifH) were amplified and fused with a hexahistidine His₆ tag as follows. The genes for hypothetical proteins BT4389 from *B. thetaiotaomicron* and YifH from *E. coli* were PCR-amplified from the genomic DNA using the following oligonucleotide primers: BT4389Fndel, 5'-ACA TAT GAT TTC AAT CAC AAA AGA TAA AAG-3'; BT4389RKpn, 5'-TGG TAC CTT AGT GGT GGT GGT GGT GGT GTT ATT TAT GAA TCA TGA TGA-3' (for BT4389); DUF152Ndel forward, 5'-ACA TAT GAG TAA GCT GAT TGT CCC GC-3'; DUF152XhoI reverse, 5'-TCT CGA GCC AAA TGA AAC TTG CCA TAC G-3' (for yfH). Amplification condition was as follows: 95°C for 120 s; 30 cycles of 95°C for 45 s, 50°C for 60 s, and 72°C for 120 s; and 72°C for 500 s. The ~730–800-bp PCR products were purified through agarose gel electrophoresis, extracted with a QiaExII gel extraction kit (Qiagen), cloned into the pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen) as recommended by the supplier, and electroporated into *E. coli* DH5 α electrocompetent cells (Invitrogen). Positive clones were selected on LB agar supplemented with kanamycin (50 $\mu\text{g}/\text{ml}$) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; 5 mg/ml). Plasmids pCRBT4389 and pCRYfH harboring the PCR-amplified DNA fragments were isolated using a plasmid mini kit from Qiagen and sequenced using M13 and rM13 oligonucleotide primers. Fragments containing the coding sequences for BT4389 and YifH were excised from these plasmids with endonucleases NdeI and KpnI (for BT4389) and NdeI and XhoI (for YifH), gel-purified as described above, ligated (14°C for 16 h, T4 DNA ligase from New England Biolabs) to the pET-31b plasmid vector (Novagen) predigested with the same endonucleases, and dephosphorylated at 37°C for 1 h with shrimp alkaline phosphatase (Roche Applied Science). Ligation mixtures were transformed into *E. coli* DH5 α non-expression electrocompetent cells (Invitrogen) plated on LB agar supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. Thereafter, the clones were controlled for correct inserts, and the plasmids were extracted and transferred to the expression *E. coli* hosts BL21(DE3) Origami (pLysS) (Novagen). The culture condi-

tions and protein purification were the same as for RL5 laccase (see above).

N-terminal Sequencing—Purified protein (20 ng) was run on a denaturing SDS-PAGE (10–15% polyacrylamide) as described by Laemmli (31), and the protein band was blotted onto a polyvinylidene difluoride membrane (Millipore) by means of a semidry blot transfer apparatus according to the instructions of the manufacturer. The membrane was stained with Coomassie Brilliant Blue R250 and destained with 40% methanol, 10% acetic acid, and the stained bands were excised and processed for N-terminal amino acid sequencing.

ICP-MS—The metal ion content of RL5 wild type and variants was determined by dilution of 50 μg of enzyme with 5 ml of 0.5% (v/v) HNO_3 to digest the protein and release the metal ions, and the solutions were analyzed with a PerkinElmer Life Sciences ICP-MS (model PE ELAN 6100 DRC).

EPR Experiments on Copper Laccase Samples—EPR measurements were carried out with a Bruker ER200D instrument operating in the X-band ($\nu \approx 9.6$ GHz) using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) standard (in a T-type double cavity) for frequency calibration. Portions of sample in potassium phosphate buffer were introduced into a spectroscopic quartz probe cell. The spectra were recorded at 77 K and were typically performed at 19.5-milliwatt microwave power (no signal saturation was apparent in independent experiments up to 40 milliwatts), 100 kHz modulation frequency, 2 G modulation amplitude, 40 ms time constant, and 1×10^5 receiver gain. Computer simulation was employed to determine EPR parameters.

Circular Dichroism—Circular dichroism (CD) spectra of proteins (2 mg/ml in 100 mM sodium acetate, pH 4.5, 20°C) were measured under N_2 atmosphere with a Jasco J-720 spectropolarimeter, employing a 0.01-cm cell. The enzyme concentration was determined using the absorption band at 280 nm ($\epsilon_{280} = 9970$, 27,390 and 37,930 $\text{M}^{-1} \text{cm}^{-1}$ for RL5, BT4389, and YifH, respectively). The molar absorptivity (ϵ) at 280 nm was determined by amino acid analysis (www.expasy.org/tools/protparam).

Assays with Purified Laccases—The standard spectrophotometric laccase assay was performed at 530 nm using SGZ as the oxidation substrate ($\epsilon_{530} = 64,000 \text{ M}^{-1} \text{cm}^{-1}$) in a thermostated spectrophotometer (PerkinElmer Life Sciences) at 40°C in 100 mM sodium acetate buffer, pH 4.5 (1 ml). The reaction was started by adding SGZ from a stock solution in methanol to a final concentration of 20 μM . Alternative substrates for measurement of laccase activity were ABTS ($\epsilon_{420} = 38,000 \text{ M}^{-1} \text{cm}^{-1}$), DMP ($\epsilon_{468} = 14,800 \text{ M}^{-1} \text{cm}^{-1}$), tetramethylbenzidine ($\epsilon_{655 \text{ nm}} = 39,000 \text{ M}^{-1} \text{cm}^{-1}$), veratryl alcohol ($\epsilon_{310} = 9,000 \text{ M}^{-1} \text{cm}^{-1}$), guaiacol ($\epsilon_{470} = 26,600 \text{ M}^{-1} \text{cm}^{-1}$), 3,4-dimethoxybenzyl alcohol ($\epsilon_{310} = 9,500 \text{ M}^{-1} \text{cm}^{-1}$), and 4-methoxybenzyl alcohol ($\epsilon_{500} = 38,000 \text{ M}^{-1} \text{cm}^{-1}$), all used at a final concentration of 1 mM. The consumption of redox mediators 1-HBT and violuric acid (1 mM) was monitored by UV absorption at 280 nm. A standard mixture of 1-HBT and violuric acid was used for identification and quantification of the eluted compounds. Oxidation of phenol red (75 μM), in the presence or absence of redox mediators (10 mM 1-HBT and violuric acid), was monitored by the decrease in absorbance at 432 nm. Activity-pH and activity-temperature relationships were determined by incubating

Novel Polyphenol Oxidase from Rumen Metagenome

enzyme-substrate mixtures at different pH values and constant temperature (40 °C) and at different temperatures (15–80 °C) and constant pH (4.5), respectively. Catalytic constants were derived by fitting the experimental data to the Michaelis-Menten model. Control measurements without enzyme were carried out to correct for any chemical oxidation of the substrates.

Decolorization of poly-R 478 (0.025 w/v) in the presence or absence of 1-HBT (0.9 mM) was determined spectrophotometrically (520 nm) at room temperature in 100 mM sodium phosphate buffer, pH 4.5. Unless otherwise indicated, the assays were routinely measured with 1.0 units of purified enzyme (referred to ABTS oxidation) over an assay time of 3500 min.

Electrochemical Analysis—Electrochemical measurements were performed with a BAS CV-50W voltammetric analyzer. For gold surface experiments, a gold BAS disc electrode was used. To clean the electrode, it was firstly immersed in “piranha” solution, containing 1 part hydrogen peroxide (33% v/v) and 3 parts sulfuric acid (98%), for 5 min, rinsed thoroughly with Milli Q water, polished with Buehler® micropolish γ -alumina 3, 0.05-micron size, for 5 min on a polishing cloth, immersed in Milli Q water, subjected to sonication for 15 min, and treated by sulfuric acid oxidization cyclic voltammetry for 30 min. It was then rinsed three times with Milli Q water, polished again for 5 min with the same alumina powder, sonicated for 15 min, and finally immersed in a reductive 0.5 M NaOH solution for 30 min and subsequently rinsed with Milli Q water. The laccase was immobilized on the gold electrode by placing 15 μ l of the enzyme solution (5 mg/ml) on the electrode, which was then covered to avoid evaporation. A second drop was added after 90 min. After 180 min from the first addition, the laccase-modified gold electrode was measured in 100 mM phosphate buffer, pH 6.0, in the presence of oxygen. For measurements under anaerobic conditions, N₂ was bubbled through the electrochemical cell for 15 min. Enzyme immobilization on a BAS glassy carbon electrode (polished with a graphite-polishing cloth for 5 min until a mirror-like surface was obtained, and rinsed thoroughly with water) involved placing 20 μ l of the laccase solution (5 mg/ml) on the glassy carbon surface for 20 min. All electrochemical measurements made with the laccase-gold electrode were repeated with the laccase-carbon electrode.

Molecular Modeling—A search in the Protein Data Bank for proteins of known structure homologous to RL5 yielded two entries, 1T8H from *Bacillus stearothermophilus* and 1RV9 from *Neisseria meningitidis* (not published), which exhibited 33.2 and 31.8% sequence identity with RL5, respectively. Both are bacterial proteins of unknown function. We chose 1T8H as a suitable template for modeling. A structural alignment of RL5 and 1T8H sequences was obtained with GenTHREADER (34) and used to retrieve a RL5 model from the Swiss-Model server (35, 36). A Ramachandran plot of the predicted RL5 structure yielded 92% of the residues in favored regions and 7% in allowed regions, which is indicative of a model of good quality.

RESULTS AND DISCUSSION

RL5 Protein Belongs to a New Family of Polyphenol Oxidases

A clone designated pBKRL5 containing the gene of a novel enzyme possessing laccase activity, RL5 (EMBL/DDBJ/

GenBank™ accession number AM269758), was identified by screening a bacteriophage λ -based metagenome library of bovine rumen microflora on indicator plates supplemented with syringaldazine (for details see “Experimental Procedures”). The sequence analysis of the 5,596-bp cloned fragment predicted three open reading frames (ORFs) using the GeneMark.hmm tool for gene prediction in prokaryotes (37). The first ORF (876 bp, positions 1,473–2,348) encodes a putative 291-amino acid protein with a predicted molecular mass of 32,240 Da. This polypeptide is very hydrophilic, with an estimated pI of 4.97, and contains only one potential transmembrane domain. The predicted ORF2 encodes a product of 262 amino acids, with a deduced molecular mass of 28,282 kDa and an estimated pI value of 5.29, exhibiting typical cytosolic features. The third ORF is located 72 bp downstream of the ATG codon of ORF2, and its deduced product, a 370-amino acid polypeptide with an isoelectric point of 6.46, includes several helix-turn-helix motifs typical of DNA-binding proteins.

Sequence analysis through BlastP (38) of the deduced polypeptide sequences of the ORF1–3 gene products failed to find any significant similarity to known laccases or polyphenol oxidases. The deduced product of ORF1 belonged to the family of zinc-peptidases involved in metal-dependent peptide bond cleavage and exhibited high similarity to a predicted metalloprotease of *Cytophaga hutchinsonii* (42% identity and 62% similarity) (supplemental Fig. S1A). The deduced product of ORF3 showed high homology to DNA repair ATPases (RecF) of *B. thetaiotaomicron* and *Bacteroides fragilis* (49% identity and 69% similarity) and somewhat lower homology to many other proteins of this family (supplemental Fig. S1B). The protein encoded by ORF2 exhibited a high degree of similarity to conserved hypothetical proteins of domain of unknown function 152 (DUF152) from *B. thetaiotaomicron*, *B. fragilis*, and *C. hutchinsonii* (57, 53, and 52% similarity, respectively; Fig. 1). We suspected that ORF2 encoded the RL5 laccase, and to prove this supposition, we produced it as a fusion with a hexahistidine (His₆) tag at the C terminus and investigated its biochemical properties (see below).

Laccase RL5 Shows Unusually High Oxidative Capability over a Broad pH Range

An active C-terminal His₆-RL5 laccase fusion protein was expressed in *E. coli* (for details see “Experimental Procedures”), and 0.9 mg of pure RL5 laccase/g wet weight was recovered using a one-step purification method involving metal-chelating chromatography. The N-terminal sequence of the purified protein was determined to be MIELEKLDFAKSVGE, which corresponds to amino acid residues 1–17 of the *in silico* translated sequence of the RL5 laccase gene (ORF2).

Subunit Composition—Native PAGE and gel filtration of the purified protein revealed a native molecular mass of RL5 to be about 57 kDa. Because its theoretical monomer molecular mass is 28,282 Da, the RL5 has thus a dimeric quaternary structure. The monomer molecular mass of RL5 (~28 kDa) is the lowest one among the bacterial, plant, and fungal laccases described thus far (50–110 kDa; see Refs. 5 and 16), with the smallest one, from *Streptomyces coelicor*, having a monomeric and dimeric molecular mass of 36 and 69 kDa, respectively (39).

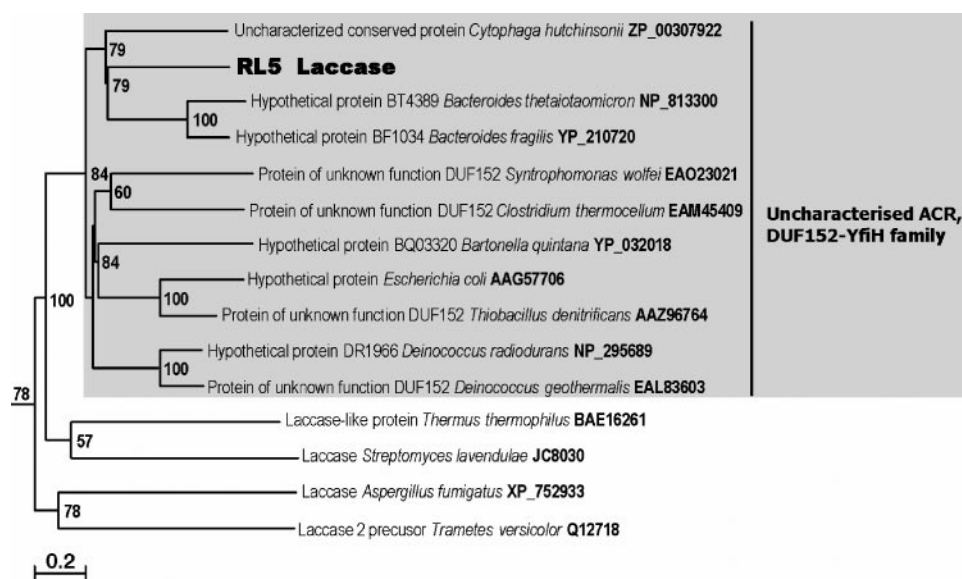


FIGURE 1. RL5 protein phylogeny. A rooted phylogenetic tree, based on an alignment of the laccase encoded by ORF2 with representatives of DUF152 proteins, was found to be homologous by Fasta 3.3 homology searches in the UniProt protein data base (release 2.3) with the BLOSUM50 matrix. Multiple sequence alignments were made using the MacVector 7.2.2 software (Accelrys, San Diego, CA) with the BLOSUM matrix and open and extended gap penalties of 10.0 and 0.05, respectively. The alignment file was then analyzed with the same software for calculation of distance matrix. Neighbor joining and Poisson correction of distances were used to construct the phylogenetic trees. The bar represents 0.2 changes/amino acid.

TABLE 1
Oxidative reactions catalyzed by RL5 laccase

Substrate	Wavelength	Relative activity ^a
	nm	%
ABTS	420	100
SGZ	530	880
DMP	468	815
4-Methoxybenzyl alcohol	500	350
Veratryl alcohol	310	465
Guaiacol	500	433
Tetramethylbenzidine	655	390
Phenol red ^b	432	17
1-HBT	280	4.0
3,4-Dimethoxybenzyl alcohol	500	— ^c
Violuric acid	280	— ^c

^a The reaction mixture consisted of 100 mM sodium acetate (pH 4.5) and 1 mM substrate (except for SGZ and phenol red, which were added to final concentrations of 25 and 75 μ M, respectively). 2 μ l of the enzyme sample (0.8 mg/ml) was added to the 1000- μ l reaction mixture in a 1-ml cuvette, and the increase or decrease in absorbance at the indicated wavelength was measured at 40 °C. Relative activity was defined as the percent increase in absorbance compared to that obtained with ABTS, which was taken as 100% (72 units/mg).

^b Oxidation of phenol red was similar in the presence or absence of redox mediators (1-HBT and violuric acid).

^c No reaction product was detected.

Copper Content—ICP-MS analysis of the metal content of recombinant RL5 protein revealed that it contains 4.0 ± 0.19 mol of copper per monomer. This suggests that the RL5 enzyme, as is the case for all well characterized laccases (5), contains one T1 copper and a T2/T3 trinuclear cluster (see below). To assess the possibility that the C-terminal His₆ tag affects the copper content, which becomes attached artificially to RL5 protein during enzyme purification/handling, its content was measured in recombinant protein without the His₆ tag. It was found, using standard purification protocols, that the recombinant protein produced without the histidine tag had the same stoichiometry of 4 mol of copper/molecule, and therefore the C-terminal His tag did not affect copper binding stoichiometry.

Kinetic Properties—The substrate preference of the RL5 laccase was as follows: SGZ > DMP > veratryl alcohol > > guaiacol > 4-methoxybenzyl alcohol >> ABTS (Table 1). 1-HBT and phenol red, an unusual substrate for laccase because of its high redox potential, were also oxidized. However, 3,4-dimethoxybenzyl alcohol and violuric acid were not oxidized by RL5.

The kinetic parameters, K_m , k_{cat} and k_{cat}/K_m , of the laccase were determined with a series of three common laccase substrates in the concentration range of 0 to 1 mM (Table 2). Steady-state kinetic measurements performed at 40 °C, pH 4.5, in the presence of 14 nM protein, yielded K_m values of 26, 0.43, and 0.45 μ M, and k_{cat} values of 1,080, 39,810, and 70,440 min^{−1} for ABTS, SGZ, and DMP, respectively. The K_m values were ~5 times lower than those reported for similar enzymes,

and the k_{cat} values were up to 40 times higher; moreover, the k_{cat}/K_m ratios of the RL5 laccase greatly exceeded the published values for the oxidation of ABTS (up to 300 min^{−1} μ M^{−1}) and especially those for SGZ (up to 9,700 min^{−1} μ M^{−1}) and DMP (up to 15,000 min^{−1} μ M^{−1}) (see examples in Refs. 5 and 40–42).

pH and Temperature Optima—The activity of RL5 with SGZ was very high over a broad pH range from 4.0 to 9.0, with an optimum at 4.0–5.0, and exhibited more than 70% activity at pH 3.5 and 9.5 (supplemental Fig. S2A). Moreover, at these pH values the enzyme was quite stable (supplemental Fig. S2A, inset). Phenol oxidase activity has previously been measured at acidic (3.0–5.0) or neutral/slightly alkaline (7.0–8.0) pH values (40). Although few laccases have been shown to be active at alkaline pH values (up to pH 9.2) those did not exhibit any significant activity below pH 6.0 (18, 38). The activity of RL5 increased with temperature, up to a maximum at ~60 °C (supplemental Fig. S2B), and the enzyme was fully stable at this temperature (supplemental Fig. S2B, inset). It should be mentioned that the bacterial and fungal laccases usually have optimal temperatures for activity between 30 and 60 °C (14, 18).

Redox Potential of T1 Site Is in the Range of Fungal Laccases

Electrochemical measurements were performed on the RL5 laccase immobilized on BAS glassy carbon and gold electrodes, which preferentially bind the enzyme in different orientations; the carbon electrode presents hydroxyl groups on its surface, resembling a laccase substrate-like structure, which favors immobilization of the enzyme with its hydrophilic active site facing the electrode surface, whereas the gold electrode provides a hydrophobic surface that repels the hydrophilic surface domains of the enzyme and favors binding of the hydrophobic domains. The different orientations of the enzyme were indeed correlated with differences in the

electrochemical measurements made. The activity measured with the gold electrode with immobilized RL5 in the presence of DMP and oxygen was 0.38 μA , whereas in the absence of oxygen it was of 0.69 μA (Fig. 2A). It is therefore likely that in the presence of oxygen the enzyme takes approximately half of the electrons from the electrode for reduction of the substrate, and the other half from oxygen, whereas in its absence, all the electrons are taken from the electrode, resulting in the higher electrochemical activity measured. The activity measured with the carbon electrode in the presence of oxygen was 2.8 μA (Fig. 2B). This suggests that 93% of the electrons were taken from the electrode, either because of the different enzyme orientation or because of immobilization of RL5 on the carbon electrode, which resulted in higher amounts (~ 7 -fold) of active laccase. In the absence of substrate, electroreduction of oxygen by the laccase-immobilized gold electrode began at +250 mV (Fig.

2C), whereas in the case of the glassy carbon electrode it began at +500 mV (Fig. 2D).

Using the carbon electrode, we also determined the oxidation potential of the T2 copper site, +500 *versus* normal hydrogen electrode (NHE). In the absence of oxygen, the oxidation potential of the T1 copper site was 740 mV *versus* NHE. When oxygen concentration was restored, the three potential oxidation peaks were observed: the T1 site of 745 mV *versus* NHE was the highest; the T3 site of 500 mV *versus* NHE had two copper ions, and thus it was the one with a higher area; and the T2 site had 400 mV *versus* NHE. The redox potential of T1 site was in the range of those of fungal laccases, *i.e.* 480–785 mV (5, 41, 42), although the genome fragment carrying the gene for RL5 is quite similar to that of a prokaryote, *B. thetaiotaomicron* (see below). However, the redox potentials of the T2 and T3 sites were close to the common values of ~ 400 mV *versus* NHE observed for both low and high potential laccases (42).

TABLE 2

Kinetic parameters

Reaction conditions were: [enzyme]₀ = 14 nM [substrate] ranging from 0 to 1 mM, 100 sodium acetate, pH 4.5, *T* = 40 °C. Kinetic parameters were calculated as described under "Experimental Procedures."

Enzyme	Substrate	k_{cat} min^{-1}	K_m μM	k_{cat}/K_m $\text{min}^{-1} \mu\text{M}^{-1}$
RL5	ABTS	1,080	26	41
	SGZ	39,810	0.43	92,580
	DMP	70,440	0.45	156,640
BT4389	ABTS	13,000	10.0	1,300
	SGZ	33,300	0.83	4,010
YifH	ABTS	1,450	23	63
	SGZ	21,720	1.10	19,750

Tentative Identification of Residues Acting as Copper Ligands in RL5

Inspection of the RL5 polypeptide sequence revealed that it does not possess the typical conserved histidine residues that usually coordinate the copper atoms of both T1 and the trinuclear clusters in laccases (see Fig. 3). Moreover, other important residues, such as Asp ligands important for H bonds in the active site (see Ref. 39), are also absent in RL5 polypeptide (Fig. 3). To identify the copper ligands in RL5, we used site-directed mutagenesis coupled with a model-driven rational

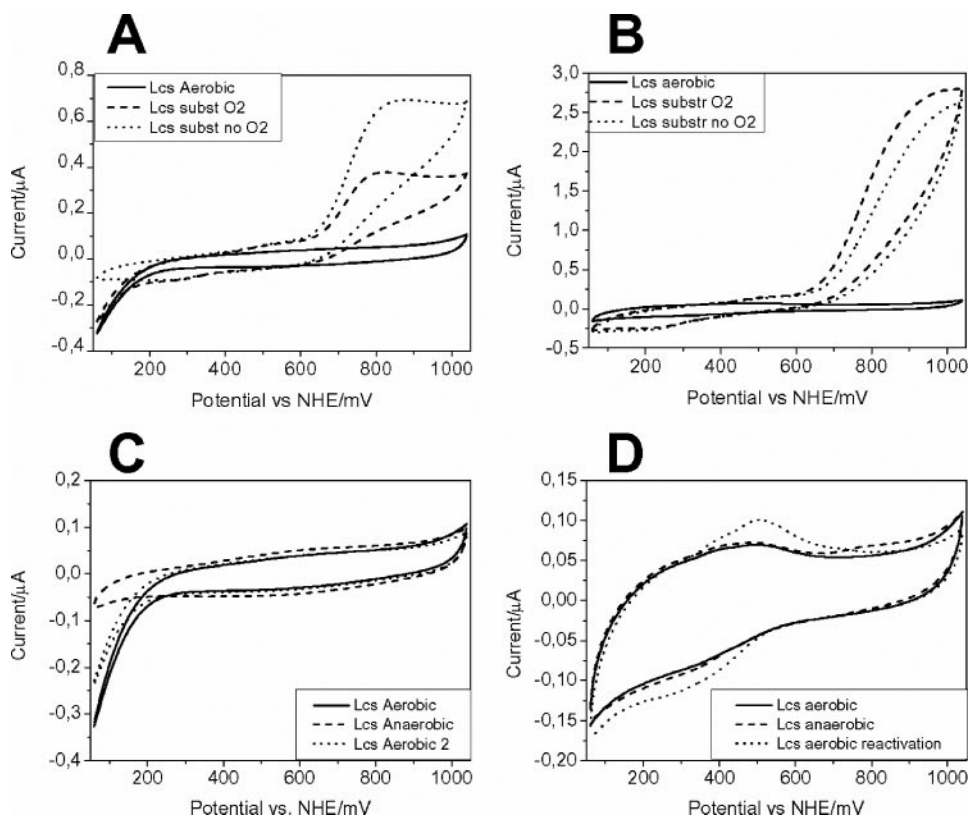


FIGURE 2. Electrochemical properties of RL5. Shown is an analysis of the electrochemical properties of electrode-immobilized enzyme on gold (A and C) or glassy carbon (B and D) electrodes in the presence of DMP at 10 mV/s (A and B) or in the absence of substrate (C and D).

design followed by activity and secondary structure determinations. We first replaced all six histidine residues in the protein, His⁷³, His¹³⁵, His¹⁹⁰, His²⁰⁷, His²³³, and His²³⁹, with Ala, and found that for the histidine to alanine substitutions (H73A, H135A, and H233A) 1.0 ± 0.2 of Cu/mol of protein structure (Fig. 4A) was removed with no substantial changes in the secondary structure by circular dichroism (not shown). As expected, none of the mutant proteins containing those mutations exhibited catalytic activity (Fig. 4B). However, although mutations at His¹⁹⁰, His²⁰⁷, and His²³⁹ took up about 3 copper atoms/molecule, they caused major changes in the secondary structure (not shown) and activity (Fig. 4B), and therefore the function of these residues as copper ligands could not be assessed directly.

To identify other potential residues involved in copper coordination, we generated a three-dimensional model of RL5 (Fig. 5) based on alignment of its sequence with that of proteins 1T8H from *B. stearrowthermophilus* and 1RV9 from *N. meningitidis* (not

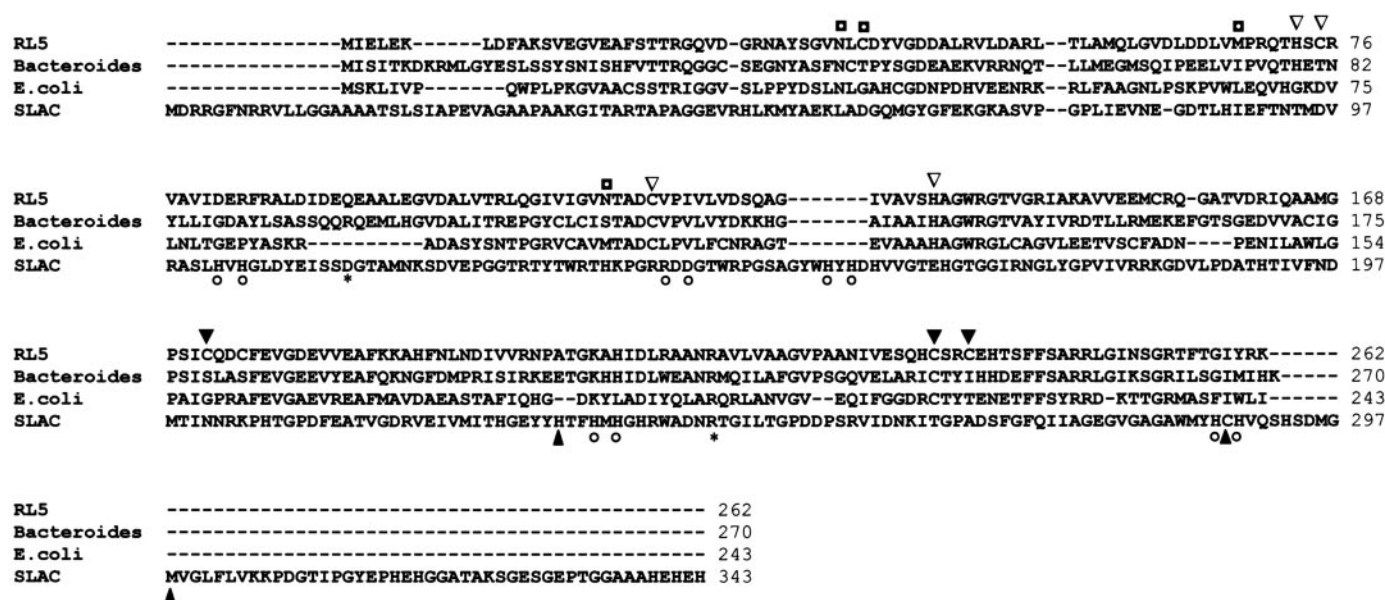


FIGURE 3. Multiple sequence alignment and identification of copper ligands for the RL5 laccase. Multiple sequence alignments were made using a ClustalW online tool for the protein sequences. *RL5*, laccase from rumen metagenome (this work); *Bacteroides*, hypothetical protein BT4389 from *B. thetaiotaomicron* (GenBank™ accession number NP_81330); *E. coli*, hypothetical protein YifH from *E. coli* (GenBank™ accession number AAG57706); *SLAC*, small laccase from *S. coelicolor* (see Ref. 39). *, important ligands for H bonds in active site of *SLAC*; ○, ligands to the trinuclear cluster in *SLAC*; ▲, ligands to the type I copper site (Met²⁹⁸ is the axial position for T1 site) in *SLAC*; ▽, ▽, □, amino acid residues belonging to the three copper sites identified in the *RL5* enzyme.

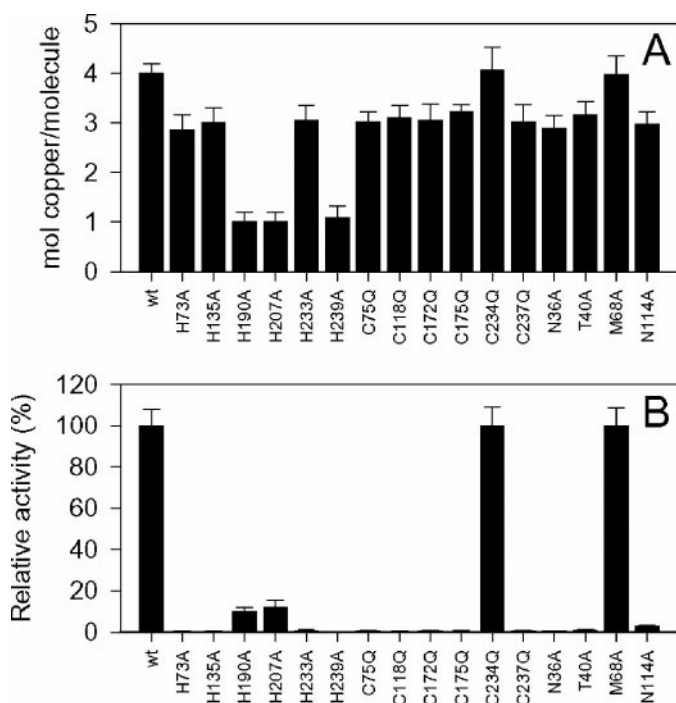


FIGURE 4. Copper content (A) and oxidation activity with ABTS (B) of His to Ala and Cys to Gln mutants of *RL5*. Copper content was determined using the PerkinElmer Life Sciences ICP-MS. *RL5* wild type and mutant variants were purified using a nickel-Sepharose column after expression with a C-terminal His₆ tag.

published), which exhibited 33.2 and 31.8% sequence identity with *RL5*, respectively. Both are bacterial proteins of unknown function. According to this model, one of the copper sites could be formed by His⁷³, Cys⁷⁵, Cys¹¹⁸, and His¹³⁵. To test whether the cysteine residues are the copper ligands, we created variants containing C75Q and C118Q mutations. In both cases one copper center was removed, which may explain why the activity was less

than 2% of that of the native enzyme (Fig. 4B) without changes in the secondary structure. This is consistent with the model prediction that the Cys⁷⁵ and Cys¹¹⁸ residues should serve as copper ligands together with His⁷³ and His¹³⁵.

The model further suggests two additional potential copper ligand centers, the first one formed by Cys¹⁷², Cys¹⁷⁵, Cys²³⁴, and Cys²³⁷ and the second one by Asn³⁶, Tyr⁴⁰, Met⁶⁸, and Asn¹¹⁴. On the basis of the model, we subsequently depleted putative copper ligands, thus generating single mutant proteins lacking copper atoms. As shown in Fig. 4B, none of the mutants except those at Cys²³⁴ and Met⁶⁸ were enzymatically active (Fig. 4B). This finding was in agreement with their lower copper content (3 versus 4 copper atoms/molecule) (Fig. 4A). We have thus identified three of four copper sites and their copper ligands. Further studies will obviously be needed to identify other copper ligands in the novel multicopper oxidase *RL5*.

Gene Arrangement in the Fragment Carrying the Gene for *RL5* Is Similar to That of the *B. thetaiotaomicron* Genome

Even though the analysis of metagenome libraries can be an extraordinarily powerful tool in revealing new functional diversity, a major constraint is the linking of newly discovered proteins to the organisms expressing them in the environment, because the small genomic fragments cloned in the libraries usually lack genes exhibiting adequate taxonomic information. In the absence of a linked, highly conserved gene such as those encoding 16 S rRNA, DNA polymerase, or RecA, codon usage and genomic context as revealed by sequence alignment of gene clusters with genomes in the data bases can be used to derive phylogenetic inferences, although these are less robust than those based on conserved genes. As indicated above, the genomic fragment carrying the *RL5* laccase gene also contains putative metalloprotease and *recF* genes highly similar to either a *Bacteroides* species or *C. hutchinsonii*, bacteria belonging to the diverse

Novel Polyphenol Oxidase from Rumen Metagenome

Cytophaga-Flexibacter-Bacteroides (CFB) phylum. Because CFB is one of the three dominant bacterial phyla of the rumen ecosystem, the probability of cloning a genome fragment of one of its members was in our case very high.

To obtain further information on the origin of the RL5 fragment, we analyzed the region immediately downstream of the

putative *recF* gene and found two additional ORFs transcribed in the same orientation as *recF*. The proteins deduced from ORF4 and ORF5 were 62 and 60% similar to a putative zinc ribbon-containing RNA-binding protein of the COG5512 family and a thioesterase from *B. thetaiotaomicron*, respectively. Moreover, the arrangement of ORFs 1–4 on the cloned genome fragment was

similar to that in the *B. thetaiotaomicron* genome (Fig. 6), namely the putative metalloprotease and conserved hypothetical DUF152 genes of *B. thetaiotaomicron* form a cluster of oppositely oriented ORFs, as is the case for the cloned fragment; the *recF* and COG5512 genes also occur together in the *B. thetaiotaomicron* genome and the cloned fragment. However, although these two gene clusters are present together in the RL5 fragment, they are separated by almost 350,000 bp in the *B. thetaiotaomicron* genome.

Conserved Hypothetical Proteins DUF152 from *B. thetaiotaomicron* and *E. coli* Exhibit Structural, Catalytic, and Spectral Properties Similar to Multicopper Oxidases

The present study has revealed that RL5 protein may become the first characterized member of a new family of polyphenol oxidases with laccase activity. To prove that this is not a coincidence, and that other homologous proteins are multicopper proteins with laccase activity, the hypothetical proteins from *B. thetaiotaomicron* (BT4389) and from *E. coli*

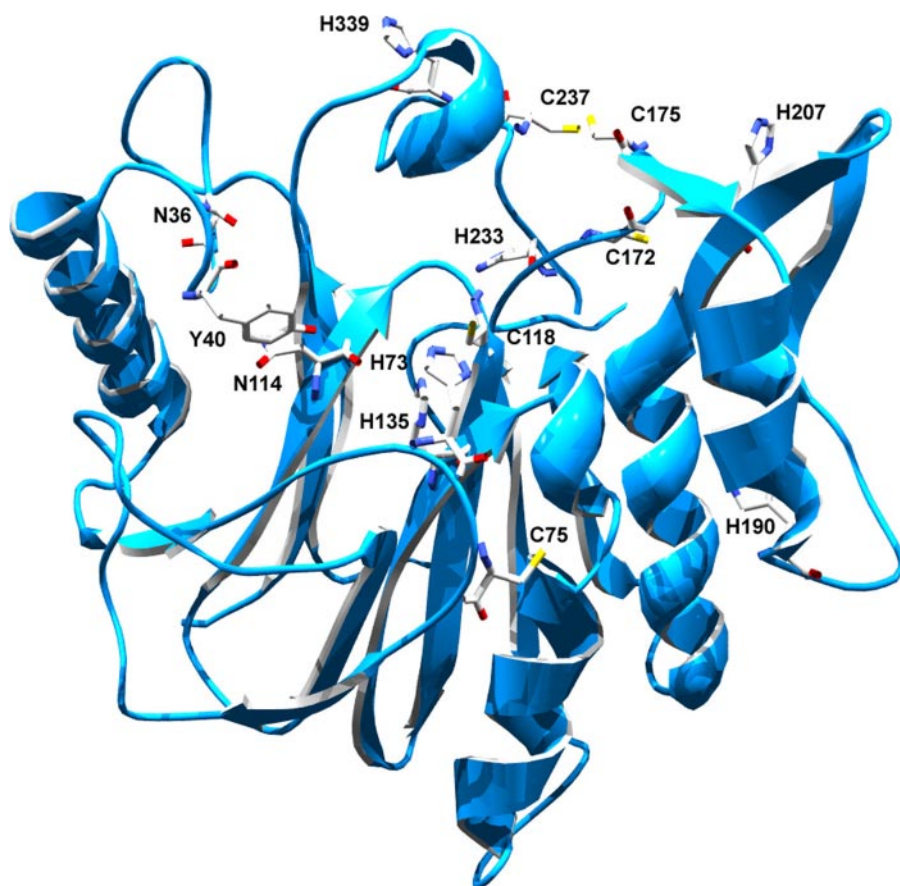


FIGURE 5. **Model of the RL5 laccase.** The overall three-dimensional structure obtained by homology modeling and the location of His residues subjected to mutagenesis are shown.

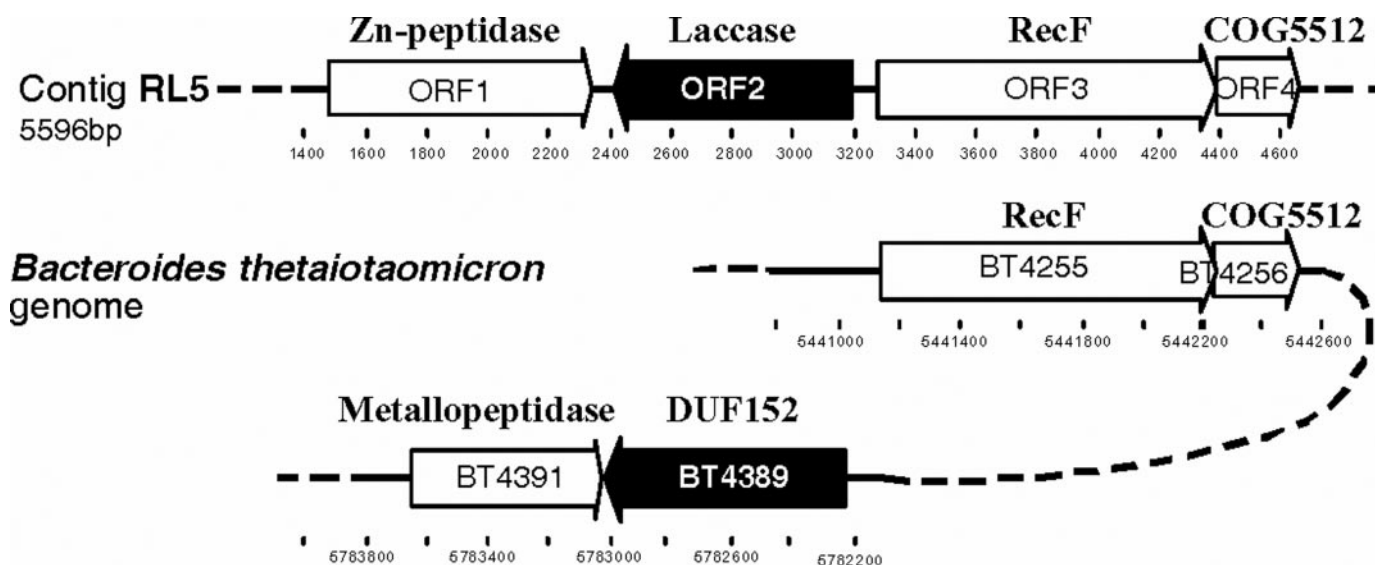


FIGURE 6. **Genomic organization of the region of RL5 gene.** The arrangement of the RL5 gene and other ORFs is shown and compared with similar region of the *B. thetaiotaomicron* genome. The RL5 laccase and DUF152 genes are highlighted on a black background.

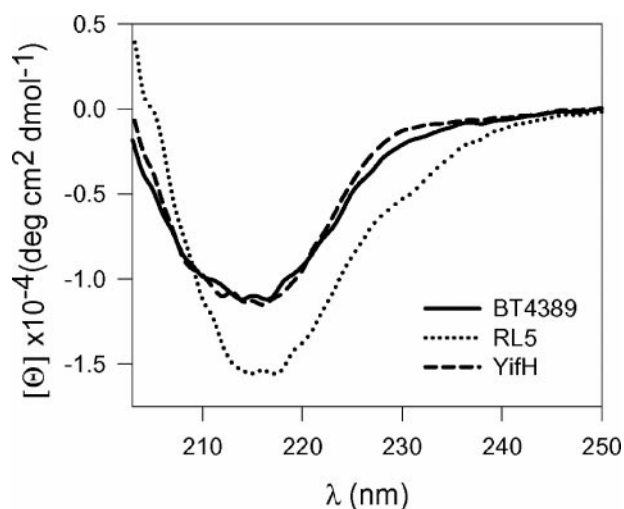


FIGURE 7. CD spectra of the RL5 (rumen metagenome), YifH (*E. coli*), and BT4389 (*B. thetaioimicon*) proteins.

(yifH) (GenBankTM accession numbers ND_81330 and AAG57706, respectively) harboring the domain of unknown function DUF152 were produced as a fusion with a His₆ tag at the C terminus, purified, and partially characterized after expression in *E. coli*. Both proteins were selected because the polypeptide sequences of BT4389 and YifH have 57 and 68% similarity to RL5.

Subunit Composition and Copper Content—Purified BT4389 and YifH yielded single protein bands on 12% SDS-PAGE (not shown) with molecular masses of about 30 kDa (predicted molecular mass 30,118 Da and pI 5.84; 270-amino acid length) and 25 kDa (predicted molecular mass 26,337 Da and pI 5; 243-amino acid length), respectively. Analysis of the native proteins revealed that they existed as dimers (~60 and 55 kDa) and contained 4.0 mol of copper/monomer, similar to those values for RL5 protein.

Kinetic Parameters—The steady-state kinetic data toward various aromatic compounds was further measured. As shown in Table 2, both proteins were able to oxidize at 40 °C and pH 4.5 ABTS and SGZ, with K_m values of 10 and 0.83 for YifH and 23 and 1.10 for BT4389 enzymes, respectively. Those values are in the range of the RL5 enzyme. Remarkably, the k_{cat} values for the BT4389 enzyme were much higher than that shown by RL5 (13,000 and 33,300 min⁻¹ for ABTS and SGZ, respectively), whereas that shown by YifH was in the same order (1450 and 21,720 min⁻¹ for ABTS and SGZ, respectively). Taken together, these results unambiguously confirm that both hypothetical proteins are highly efficient multicopper oxidases with laccase activity.

pH and Temperature Optima—The optimum temperatures of the enzymes were 52 °C (for BT4389) and 44 °C (for YifH), and the thermal activity was maintained at more than 80% at 55 °C (for BT4389) and 50 °C (for YifH). The enzymes were optimally active at a pH ranging from 4.5 to 6.0 (for BT4389) and from 5.5 to 8.4 (for YifH), although they maintained 80% activity at pH 4.0–7.5 (for BT4389) and 5.0–9.0 (for YifH).

Electrochemical Measurements

The cyclic voltammetry measurements (supplemental Figs. S3 and S4) revealed that both YifH and BT4389 proteins are multicopper oxidases, having a redox potential of 283–300 mV (for the T2-T3 cluster) and 406–416 mV (for T1 site) *versus*

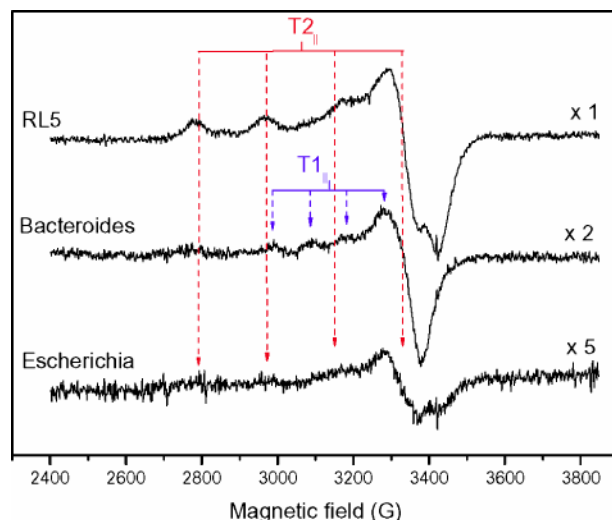


FIGURE 8. EPR spectra of the indicated samples at 77 K. The hyperfine splitting of the parallel component of the indicated signals is displayed.

NHE. Therefore, both enzymes may be defined as low potential laccases (similar to other bacterial laccases (5, 41, 42)). Both values clearly contrast with the high redox potential of RL5 (~745 mV *versus* NHE) and agree perfectly with the higher capability of RL5 to decolorize the dye poly-R compared with the YifH and BT4389 proteins (see supplemental Fig. S5). These results suggest that the copper ligands may play an important role in the differences observed in the redox potential of the three enzymes, particularly the copper ligands of the T1 sites, because this copper site defines the redox potential of polyphenol oxidases. Thus, among the 12 identified copper ligands, only four (Asn³⁶, Cys¹¹⁸, His¹³⁵, and Cys²³⁴; RL5 numbers) are conserved among the three enzymes. If this is so, the different redox potential could be ascribed to electrostatic and steric changes in the copper-binding pocket induced by the amino acid ligands. Further studies will be conducted in the near future to validate these predictions.

CD Analysis

In addition to the biochemical and electrochemical analyses, we also examined the CD spectral features of the three enzymes under investigation. As shown in Fig. 7 the spectra of RL5, YifH, and BT4389 were similar, with minima at around 216 nm indicative of typical β -sheet structures (43), which is compatible with the structures of multicopper oxidases and apparently at variance with the α/β homology model of RL5 (Fig. 5) based on the structures of 1T8H and 1RV9.

EPR Analysis

EPR spectra of the samples are displayed in Fig. 8. The spectra appear basically to be constituted by the overlapping of two Cu²⁺ signals, referred to as T1 and T2 (following similar nomenclature employed in previous analyses of samples of this type (38, 41, 44)). Both of the spectra presented an axial shape with $g_{||} = 2.235$, $A_{||} = 19.5 \times 10^{-3}$ cm⁻¹ (187 G), and $g_{\perp} = 2.052$ for T2 and $g_{||} = 2.182$, $A_{||} = 9.8 \times 10^{-3}$ cm⁻¹ (96 G), and $g_{\perp} = 2.045$ for T1 (A_{\perp} could not be determined for any of the signals because of their relatively large width in comparison with this hyperfine splitting). The main dif-

ferences between the samples appear to be related to the relative contributions of the signals. Although T2 clearly predominates in RL5 (and probably also in *Escherichia*, according to the shape of the perpendicular zone), T1 is mainly responsible for the spectrum observed for the *Bacteroides* enzyme. Differences are also apparent between the EPR parameters observed for these signals and those observed previously for analogous signals (in type 1 and type 2 copper sites) in other copper laccases (39, 41, 44), thus reflecting differences (even if subtle) in the respective chemical environments present in each case.

Concluding Remarks

In the present study we retrieved, from a bovine rumen metagenome library, and characterized a novel laccase that neither exhibited any sequence similarity to known laccases nor contained hitherto identified functional laccase motifs. It exhibited a 5-fold higher affinity than typical laccase substrates and catalysis rates 40-fold higher than those reported thus far, and it oxidized a broad spectrum of substrates over a wide pH range. RL5 is a new, high performance catalyst possessing novel structural features that may open new perspectives for structure-function studies of multicopper oxidases. Biochemical, spectroscopic, and EPR analysis revealed that two representatives of the DUF152 family (COG1496), namely BT4389 and YifH proteins from *B. thetaiotaomicron* and *E. coli*, were also laccases. This suggests that other multicopper oxidases with various substrate spectra may further be mined among the numerous representatives of DUF152/COG1496. Moreover, the gene for the RL5 enzyme was derived from a rumen microbial community, which processes the plant material at high rates. Laccases in the rumen may play an important role in ryegrass lignin digestion, so the RL5 enzyme may have biotechnological potential for exploitation in pasture-fed animals and in pasture grasses. Finally, the present study demonstrates the power and utility of activity-based metagenomics for the exploration of functional diversity space and the discovery of novel enzymes with laccase activity in a protein lacking homology to any described polyphenol oxidase.

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